



Behavioural differences between *Aphidius ervi* populations from two tritrophic systems are due to phenotypic plasticity

P. Daza-Bustamante¹, E. Fuentes-Contreras², L.C. Rodríguez¹, C.C. Figueroa^{1,3} & H.M. Niemeyer^{1,*}

¹Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile (Fax: +56 2 271-7503; E-mail: niemeyer@abulafia.ciencias.uchile.cl); ²Departamento de Producción Agrícola, Facultad de Ciencias Agrarias, Universidad de Talca, Casilla 747, Talca, Chile; ³Instituto de Ecología y Evolución, Universidad Austral de Chile, Casilla 567, Valdivia, Chile; *Author for correspondence

Accepted: August 16, 2002

Key words: *Aphidius ervi*, *Acyrtosiphon pisum*, *Sitobion avenae*, host searching behaviour, wind tunnel, aphid parasitoids, molecular markers, RAPD-PCR, mtDNA, Hymenoptera, Aphidiidae

Abstract

The Palaeoarctic parasitoid *Aphidius ervi* Haliday (Hymenoptera, Aphidiidae) parasitises legume aphids in its region of origin. In Chile, it parasitises both legume and cereal aphids. This special situation was studied at two levels: (i) the host searching behaviour of *A. ervi* from two different tritrophic systems (*Acyrtosiphon pisum* on alfalfa and *Sitobion avenae* on wheat) was investigated in dual choice tests in a wind tunnel between odours from both *A. pisum*-alfalfa host plant complex (HPC) and *S. avenae*-wheat HPC, and (ii) the genetic structure of *A. ervi* populations from both sources using molecular markers. Responses of *A. ervi* females to volatile olfactory cues emanating from *A. pisum*-alfalfa HPC and *S. avenae*-wheat HPC were significantly higher towards the HPC on which they were reared during the last generation before experimentation, regardless of the origin of the parasitoid. As previously described for this parasitoid species, oviposition experience was also of major relevance in the preferences of female parasitoids. On the other hand, variation in mitochondrial DNA segments and RAPD-PCR polymorphism using total DNA showed the absence of host-based population structure and a high genetic homogeneity between these *A. ervi* populations. These results reject the possible existence of different host-strains of this parasitoid in Chile.

Introduction

Host-searching behaviour by aphid parasitoids is a complex process which may involve genetic or innate preferences (Poppy et al., 1997; Glinwood et al., 1999a, b), the physiological state and previous experience of the parasitoid (Grasswitz & Paine, 1993a), as well as the influence of abiotic environmental conditions (Schwoerer & Völkl, 2001). In particular, the host-searching process by aphid parasitoids is strongly influenced by semiochemicals (Vet & Dicke, 1992; Turlings et al., 1993; Rutledge, 1996; Poppy et al., 1997; Powell et al., 1998), which may be released from the aphid food plants, from the host aphids, and from the interacting host aphid and aphid food

plant, known as the host plant complex (HPC) (Du et al., 1997, 1998; Guerrieri et al., 1993, 1997, 1999; Powell et al., 1998; Glinwood et al., 1999a, b; Bradburne & Mithen, 2000). Furthermore, parasitoids have been shown to change their responses to semiochemical cues as a result of experience obtained during foraging as an adult, either by responding to cues which were previously ignored or by enhancing existing responses (associative learning) (Sheehan & Shelton, 1989; Reed et al., 1995; Grasswitz & Paine, 1993b; Du et al., 1997). Additionally, female parasitoids have been shown to change their preferences as a consequence of the acquisition of chemical information during the immature stage or during emergence

from the mummy case (Wickremasinghe & van Emden, 1992; van Emden et al., 1996; Poppy et al., 1997; Storeck et al., 2000). All the above mentioned components of host-searching behaviour may influence the distribution and abundance of parasitoids associated with different HPC in the field. Such behavioural differences could produce differentiation of parasitoid subpopulations, leading to the formation of genetically different strains or biotypes associated with a particular HPC (Vaugh & Antolin, 1998).

Aphidius ervi Haliday is a Palaeoarctic oligophagous parasitoid species associated in its region of origin mainly with Macrosiphinae aphids, such as *Acyrtosiphon pisum* Harris on legumes and to a lower degree with *Macrosiphum euphorbiae* Thomas and *Aulacorthum solanii* (Kaltenbach) on other aphid food plants (Takada & Tada, 2000). Although *Sitobion avenae* (Fabricius) on cereals is a suitable host for *A. ervi*, this parasitoid has a minor relevance as an aphid bio-control agent on the cereal agroecosystem in Europe (Cameron, 1984). In Chile, where this parasitoid was introduced nearly 25 years ago (Zúñiga, 1990), it has been reported to parasitise both *A. pisum*, an aphid prevalent in alfalfa, and *S. avenae*, an aphid prevalent in wheat (Starý, 1978, 1993; Starý et al., 1993).

In order to study behavioural differences between parasitoids associated with host aphids from different agroecosystems, the present research focuses on the situation of *A. ervi* in Chile parasitising aphids on alfalfa and wheat. The influence of parasitoid origin, rearing HPC, and oviposition experience on the host-searching behaviour of this aphid parasitoid were addressed. In particular, parasitoids collected in the field and subsequently reared on both *A. pisum* on alfalfa and *S. avenae* on wheat, and subjected to oviposition experience on both HPC, were assayed in dual choice tests in a wind tunnel, against odours from both *A. pisum*-alfalfa HPC and *S. avenae*-wheat HPC. Furthermore, the genetic structure of parasitoid populations from both hosts were compared using two types of molecular markers.

Materials and methods

Parasitoids. *Aphidius ervi* of two origins was obtained from parasitised aphids (mummies) collected on alfalfa, *Medicago sativa* L., or wheat, *Triticum aestivum* L., at the INIA-La Platina fields in Santiago - Chile. Once emerged in the laboratory, parasitoids from *A. pisum* on alfalfa were reared on *A. pisum*

on alfalfa, and parasitoids from *S. avenae* on wheat were reared on *S. avenae* on wheat. The colonies were maintained under laboratory conditions at 20 ± 1 °C, and L14:D10 photoperiod; they were supplemented twice (at two-month intervals) with specimens brought in from the field which were allowed to intercross *ad libitum* with the existing colony. Individuals used in this study had been raised in the laboratory for at least six generations.

Parasitoids. Mated females from the colony on *A. pisum* on alfalfa were allowed to oviposit on either *A. pisum*-alfalfa HPC or *S. avenae*-wheat HPC, and mated females from the colony on *S. avenae* on wheat were allowed to oviposit either on *S. avenae*-wheat HPC or *A. pisum*-alfalfa HPC ('rearing host plant complexes' of Table 1). Hence, in the transfer experiments where a change of host plant complex occurred, parasitoids developed in the alternative host during one generation.

On a given day, parasitoids which had emerged during the previous night in one of the rearing HPC were sexed, and fed with honey solution. Females were allowed to mate overnight. Preliminary assays showed this time to be amply sufficient for mating to occur. The following morning, mated females were allowed oviposition experience for 30 min either on the same HPC of rearing or the alternative HPC (HPC: plants infested for 48 h with 25 *A. pisum* or *S. avenae* aphids, respectively), and 3 to 4 h later they were submitted to experimentation in the wind tunnel (Du et al., 1997).

Plants. The plants used as odour source for female parasitoids were: 3-week old alfalfa plants and 2-week old wheat plants which had been previously infested with 25 host aphids (*A. pisum* and *S. avenae*, respectively) during 48 h, thus constituting the host plant complex.

Wind tunnel assays. The orientation behaviour of *A. ervi* females during flight was studied in a wind tunnel constructed from a rectangular Plexiglas chamber (80 × 30 × 30 cm). The air speed for all the experiments was adjusted to 20 ± 1 cm/s, and the light intensity to 3600 lux. The walls of the wind tunnel were covered externally with thin white paper sheet to prevent the influence of other stimuli in host selection. Each female parasitoid (14 individuals per treatment) was released from a Plexiglas box (1.5 cm³) located 20 cm above the floor of the wind tunnel. The distance from release

Table 1. Responses of *Aphidius ervi* females from different origins, reared on two different host plant complexes (HPC), confronted with odours from *Acyrtosiphon pisum*-alfalfa HPC and *Sitobion avenae*-wheat HPC, in a wind tunnel. Parasitoids either did not have oviposition experience (o.e.) or had it on either of the two HPC^a

Parasitoid origin	Rearing host plant complex	Treatment	Oriented flight		Mean response time \pm s.e. (seg)
			Alfalfa HPC	Wheat HPC	
Alfalfa	<i>A. pisum</i> -alfalfa	Without o.e.	13a	0b	102.9 \pm 20.8c
		With o.e. (alfalfa-HPC)	13a	0b	37.9 \pm 10.9d
		With o.e. (wheat-HPC)	0a	11b	38.6 \pm 8.2d
	<i>S. avenae</i> -wheat	Without o.e.	0a	12b	124.1 \pm 16.6c
		With o.e. (wheat-HPC)	0a	11b	85.1 \pm 15.1d
		With o.e. (alfalfa-HPC)	13a	0b	88.6 \pm 8.3d
Wheat	<i>A. pisum</i> -alfalfa	Without o.e.	12a	0b	135.6 \pm 21.4c
		With o.e. (alfalfa-HPC)	11a	0b	102.3 \pm 21.5c
		With o.e. (wheat-HPC)	0a	13b	107.8 \pm 12.9c
	<i>S. avenae</i> -wheat	Without o.e.	0a	11b	125.8 \pm 23.5c
		With o.e. (wheat-HPC)	0a	14b	42.8 \pm 3.1d
		With o.e. (alfalfa-HPC)	12a	0b	51.6 \pm 7.4cd

^aFourteen female parasitoids were used for each treatment. In comparisons of oriented flights towards different odour sources for each origin-rearing HPC combination, values followed by the same letter are not significantly different according to χ^2 -test (significance level: $P < 0.05$). In comparisons of mean response times between different rearing HPC within each origin, values followed by the same letter are not significantly different according to Kruskal Wallis non-parametric test (significance level: $P < 0.05$). Power of performed tests with $\alpha = 0.05$, were in all cases higher than 0.95.

point to the odour sources was 35 cm. Two stimuli (*A. pisum*-alfalfa HPC and *S. avenae*-wheat HPC) were offered simultaneously to each female parasitoid, whose behaviour was observed for a maximum of 5 min after release, a time considered sufficient to elicit a response (Du et al., 1997).

Two parameters were determined: (i) the preference of parasitoids towards the stimuli offered (flight orientation), and (ii) the response time, measured from the release of the parasitoid into the wind tunnel until obtaining a response.

Pseudoreplication was avoided by following indications by Ramírez et al. (2000). The biological material (parasitoids and plants) was used only once during the experiments, and the inside walls of the wind tunnel were cleaned with 95% ethanol between replicates. The position of stimulus plants was interchanged within the wind tunnel after each experiment, in order to avoid positional bias.

A χ^2 -test was used to compare oriented flight and/or landing responses, while the mean response times were compared using a Kruskal-Wallis test.

Genetic analyses. In order to avoid a bias due to the analysis of only female parasitoids, two differentially inherited markers were used: nuclear-inherited (random amplified polymorphic DNA-polymerase chain reaction; RAPD-PCR) and maternally-inherited (mitochondrial DNA; mtDNA). These two molecular markers have been extensively used to study host specialisation and genetic variability in insects (Sunucks, 2000). RAPD-PCR has been successfully used to differentiate parasitoid strains or biotypes of parasitoids of aphids (e.g., Edwards & Hoy, 1995; Vaughn & Antolin, 1998), and several mtDNA regions have been reported as useful in the study of host specialisation (biotypes) in Hymenoptera (e.g., Sheppard et al., 1994; Simon et al., 1994; Belshaw et al., 1999).

One hundred and six individuals collected along the development of the crops (35 from wheat and 71 from alfalfa) were analysed. Sixteen additional parasitoids whose responses in a wind tunnel had been previously determined were also screened with these markers, and corresponded to seven individuals collected from wheat and nine individuals from alfalfa.

DNA isolation. DNA was extracted using the 'salting out' method described for aphids (Sunnucks & Hales, 1996), and standardised for parasitoids by increasing the proteinase K concentration and incubation period. Total DNA was resuspended in 30 μ l of ultra-pure sterile water. DNA quality was assessed by electrophoresis in 0.8% agarose gels visualised under UV light after ethidium bromide staining. The DNA samples were stored at -20°C until used.

Random amplified polymorphic DNA-PCR (RAPD-PCR) analysis. Two random decamer primers named HN9 (5'-AATCGGGCTG-3') and CFa5 (5'-TGCGGC TGAG-3') and synthesised by Gibco-BRL and Keystone Labs (Biosource International), respectively, were used to assess the genetic diversity of *A. ervi* from the two systems studied.

Different DNA dilutions, MgCl_2 concentrations, and annealing temperatures were tested before defining the final conditions employed for the amplifications. The PCR reactions were performed in a final volume of 25 μ l containing 2.5 μ l of 10X *Taq* polymerase reaction buffer (Gibco-BRL), 2.5 mM MgCl_2 , 250 μ M dNTP's, 1 μ M primer, 0.5 units of *Taq* DNA polymerase (Gibco-BRL), 5 μ l of 1:50 dilution of resuspended DNA and ultra-pure sterile water. Thermal cycles were carried out in a Perkin Elmer 9700 thermal cycler and consisted of an initial denaturation step of 5 min at 94°C , 40 cycles of 30 s at 94°C , 30 s at 36°C , and 80 s at 72°C , and a final extension step of 10 min at 72°C . The PCR products were separated by electrophoresis in 1.5% agarose gels in 1X TAE buffer, and visualised under UV light after ethidium bromide staining (Welsh & McClelland, 1990; Williams et al., 1990).

Restriction fragment length polymorphism-PCR (RFLP-PCR). Two regions of mtDNA, corresponding to segments of the cytochrome B gene and of the region between the genes for subunits I and II of cytochrome oxidase, were shown to amplify *A. ervi* DNA samples by PCR.

The amplification conditions were as described by Sheppard et al. (1994). Three μ l of total DNA were used for each PCR reaction containing 1X buffer *Taq* polymerase (Gibco-BRL), 200 μ M dNTPs, 1 μ M of each primer, 3 U *Taq* polymerase, and ultra-pure water for a final volume of 50 μ l. Thermal cycles were carried out in a Perkin Elmer 9700 thermal cycler, and consisted of an initial denaturation step of 5 min at 94°C , 40 cycles of 1 min at 94°C , 2 min at 50°C ,

and 3 min at 72°C , and a final extension step of 5 min at 72°C . The PCR products were separated by electrophoresis in 1.5% agarose gels in 1X TAE buffer, and visualised under UV light with ethidium bromide.

In order to study the variability of these mtDNA fragments between populations of *A. ervi* parasiting aphids living on wheat and alfalfa, a battery of restriction enzymes were used. Useful digestions were only obtained with *Dra*I and *Hin*fI (New England BioLabs).

The digestion reaction was performed in 50 μ l containing 15 μ l of the amplification products, 10 U of each restriction enzyme, and 1X of the restriction buffer. After overnight incubation at 37°C , the digestion was stopped by the addition of sample buffer with 10 mM EDTA pH 8.0 (Sambrook et al., 1989). The digestion products were analysed by electrophoresis in 2% agarose gels in 1X TAE buffer, and visualised under UV light after ethidium bromide staining. Each digestion fragment was sized, and patterns obtained compared at inter-individual and inter-populations levels.

Data analysis. The presence/absence of each reproducible band was interpreted as alleles (Lynch & Milligan, 1994), and allowed the characterisation of RAPD-PCR profiles for each individual parasitoid. The RAPD-PCR phenotype for each individual considered all identified bands with all decamer primers used. Given the dominant nature of RAPD-PCR markers, the absence of a band corresponds to the recessive homozygote (null alleles) but the presence of a band may correspond either to the dominant homozygote or the heterozygote (Sunnucks, 2000). The frequency of the null allele was considered equivalent to the frequency of the recessive homozygote (Vanlerberghe-Massuti & Chavigny, 1998), and the frequency of the dominant allele was estimated using the correction introduced by Lynch & Milligan (1994) for dominant markers. Allelic frequencies and the distribution of RAPD-PCR phenotypes on different host-plant combinations, were computed with the RAPDistance freeware v. 1.04 (Armstrong et al., 1994). Comparisons between the genotypic constitution of parasitoid populations from wheat and alfalfa were performed by analysing dendrograms generated with Nei genetic distances computed with the above software. The genetic structure of these populations according to the host-plant were compared using the GST estimator of structuring computed with the POPGENE v. 1.31 freeware (Yeh et al., 1999).

Results

Wind tunnel assays. Regardless of parasitoid origin and in the absence of oviposition experience, female parasitoids reared on *A. pisum*-alfalfa HPC and on *S. avenae*-wheat HPC showed a significant preference (oriented flight) for the host plant complex on which they had been reared (Table 1). This pattern was further reinforced when the female parasitoids received oviposition experience on the HPC on which they had been reared, showing a significantly lower mean response time toward the odour sources (Table 1). On the contrary, when parasitoid females were allowed to experience oviposition on the alternative HPC, the preference was strongly changed to the alternative HPC (Table 1).

Genetic variability at RAPD-PCR loci. A total of 14 and 10 polymorphic loci (bands) were identified with HN9 and CFa5 RAPD-PCR primers, respectively. The RAPD-PCR phenotypes (banding patterns) generated by each decamer primer were compared between populations. A slightly higher genetic variability was found in parasitoids from wheat with respect to those from alfalfa. Thus, primer HN9 identified 33 different RAPD-PCR phenotypes from the 35 collected individuals in wheat and 61 from 71 individuals in alfalfa, and primer CFa5 identified 18 RAPD-PCR phenotypes in wheat and 25 in alfalfa. Also, a slightly higher Nei's allelic diversity was found in parasitoids from wheat (24%) compared to those from alfalfa (21%).

No significant differences in genotypic composition were found between populations. Thus, RAPD-PCR phenotypes obtained from both primers, either individually or in combination, and grouped using Nei genetic distance between individuals, showed no differences attributable to the host-plant combination. Additionally, the neighbour joining trees generated with Nei genetic distances showed no structuring of data, indicating a high degree of relatedness between the individuals from both populations, with a Nei genetic identity over 99% between populations.

Differences in allelic structure were not detected between both populations. Since GST indexes were less than 1% for all loci studied, no allelic structuring was noted at any locus. When the analysis was performed on aphid parasitoids with a known type of flight response in the wind tunnel, no allelic or genotypic differences were found between individuals with preference for either HPC.

Variation at mitochondrial DNA. Two fragments corresponding to the gene of cytochrome B (CytB) (480 bp) and the region between genes for subunits I and II of cytochrome oxidase (COI/II) (600 bp), were amplified. After digestion of Cyt B with *Dra*I restriction enzyme, two restriction patterns were obtained. One corresponded to no digestion (labelled restriction pattern A), and the other generated fragments of 140 bp and 200 bp (pattern B), probably arising from two different restriction fragments of identical size. *Hinf*I produced no digestion in CytB. When COI/II was subjected to digestion with *Dra*I, two patterns were observed corresponding to no digestion (pattern C), and to restriction fragments of 290, 240 and 70 bp (pattern D). *Hinf*I produced two patterns corresponding to no digestion (pattern E) and to restriction fragments of 320 and 280 bp (pattern F). The restriction patterns of the parasitoid populations from wheat and alfalfa did not differ from each other (Fisher exact test, $P > 0.23$) (Table 2).

Discussion

Wind tunnel flight responses (oriented flights) of *A. ervi* females from either alfalfa or wheat were significantly stronger towards the host plant complex on which they had been reared during the last generation before the experiments. Innate preference toward alfalfa volatiles, the plant where its main host *A. pisum* is frequently found (Powell, 1994), was of minor relevance at this level, since parasitoids collected on alfalfa and maintained in the laboratory on *S. avenae*-wheat HPC significantly preferred the HPC on which they were reared. In addition, as previously described in several studies with *A. ervi*, oviposition experience was also able to change or enhance the preferences of this parasitoid (Du et al., 1996, 1997, 1998; Guerrieri et al., 1993, 1997, 1999; Powell et al., 1998).

The preference observed in the absence of oviposition experience toward the rearing HPC could be attributed to a conditioning process occurring either during larval/pupal development inside the host, or during early adult emergence from the mummy case, as has been shown for other aphid and parasitoid species. In this vein, Wickremasinghe & Van Emden (1992) demonstrated conditioning, i.e., behavioural responses acquired during larval/pupal development in contrast to adulthood (Poppy et al., 1997), in female *Aphidius rhopalosiphi* De Stefani-Pérez which, when given a choice between two wheat cultivars

Table 2. Frequencies for the restriction patterns found in *Aphidius ervi* populations from *Acyrtosiphon pisum* on alfalfa and from *Sitobion avenae* on wheat

Fragment/restriction enzyme	Restriction pattern	Wheat (26) ^a		Alfalfa (49) ^a	
		n	Frequency	n	Frequency
CytB / <i>Dra</i> I	A	5	0.19	5	0.10
	B	21	0.81	44	0.90
COI/II / <i>Dra</i> I	C	3	0.12	6	0.12
	D	23	0.88	43	0.88
COI/II / <i>Hinf</i> I	E	4	0.15	4	0.08
	F	22	0.85	45	0.92

^aNumber of individuals whose mtDNA PCR-amplified satisfactorily under the conditions employed.

showed a preference for the cultivar on which their host aphid had been reared. Likewise, Storeck et al. (2000) recently demonstrated a preference of *Aphidius colemani* Viereck for the host plant complex on which they had been reared, but this preference was not evident when the parasitoids were dissected from their mummies prior to adult emergence. In this latter case, early contact of the newly emerged parasitoid with chemical cues from the mummy case seemed to be responsible for this preference (Storeck et al., 2000).

Behavioural differences based on preferences toward the HPC on which the parasitoid had been reared, could be associated with the presence of genetically differentiated parasitoid strains or biotypes specialised on different HPC's. However, the influence of oviposition experience might provide the behavioural plasticity necessary for the utilisation of alternative HPC's. In our study, no evidence of HPC-based population structure was found at a multilocus level. Additionally, differential distribution between both populations was not detected in any allele or phenotype. These results support the hypothesis put forward by Starý et al. (1993) and Starý (1993) to account for the presence of *A. ervi* in both legume and cereal crops in Chile. Perennial legumes would serve as year-round reservoirs for parasitoids, particularly during the period when wheat is not available (end of summer, autumn and part of winter). When the vegetative period of wheat starts (late winter and spring), migration of *A. ervi* would occur from legumes to cereals and also from cereals to legumes, maintaining populations of parasitoids on both crops. A consequence of these migrations would be that *A. ervi* populations on legumes and cereals should not be genetically different (Starý et al., 1993; Starý, 1993).

In a similar study system, the parasitoid *Diaretiella rapae* (M'Intosh) is known to be associated mainly with the cabbage aphid *Brevicoryne brassicae* (L.) on cruciferous plants, but the Russian wheat aphid *Diuraphis noxia* (Kurdjumov) on cereals is also a suitable alternative host (Reed et al., 1995; Vaughn et al., 1996). Innate preferences, preemergence experience (conditioning) and adult oviposition experience have been found for *D. rapae* on *B. brassicae* on crucifer plants (Sheehan & Shelton, 1989; Reed et al., 1995; Vaughn et al., 1996), which was strongly mediated by semiochemicals characteristic of this plant family (Bradburne & Mithen, 2000). These behavioural differences were associated with a significant genetic structure among subpopulations of *D. rapae* on different HPC's (Vaughn & Antolin, 1998). The smaller spatial scale of our field survey may preclude the detection of genetic structuring, although for *D. rapae* low dispersal rates resulted in strong genetic structure at spatial scales of less than 1.0 km (Vaughn & Antolin, 1998).

The genetic homogeneity of *A. ervi* populations from both HPC is not inconsistent with wind tunnel results where different preferences were shown by *A. ervi* from different origins, since these latter may be attributed to larval, pupal, or early adult learning subsequently modified by parasitoid associative learning based on oviposition experience. These results suggest that volatile cues from HPC's play an important role in determining the host preferences shown by *A. ervi* parasitoids during the host-selection process, and that the exposure and foraging experiences on other HPC's can rapidly change these preferences, providing behavioural plasticity as a consequence of a learning process. This behavioural plasticity allows parasitoids

to make optimal use of the prevailing foraging opportunities in relation to the relative availability of different host species, within different habitat patches which are likely to change with time, particularly within agricultural ecosystems (Powell et al., 1998).

Acknowledgements

This work was supported by FONDECYT Project No. 1990711, and the Presidential Chair in Science awarded to HMN. PD-B and LCR are grateful to LANBIO (Latin American Network for Research in Bioactive Natural Compounds) for fellowships during the development of this work. This research is part of the activities of the Center for Advanced Studies in Ecology and Research in Biodiversity funded by the Millennium Science Initiative (P099-103-F ICM).

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